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Evaluation of the contribution of the renal capsule and cortex to kidney autofluorescence intensity under ultraviolet excitation

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ABSTRACT

The use of reduced nicotinamide adenine dinucleotide (NADH) fluorescence to gain metabolic information on kidneys in response to an alteration in oxygen availability has previously been experimentally demonstrated, but signal quantification has not to date been addressed. In this work the relative contribution to rat kidney autofluorescence of the capsule vs. cortex under ultraviolet excitation is determined from experimental results obtained using autofluorescence microscopy and a suitable mathematical model. The results allow for a quantitative assessment of the relative contribution of the signal originating in the metabolically active cortex as a function of capsule thickness for different wavelengths.

Keywords: lasers in medicine, fluorescence, tissues, microscopy, ultraviolet

In the cell electron transport chain, NADH (reduced nicotinamide adenine dinucleotide) is an electron carrier molecule and oxygen is the ultimate acceptor. Absence of O_2 induces a shift in the NADH redox state, which is accompanied by a change in the NADH autofluorescence intensity since its oxidized form does not fluoresce. This sensitivity gives rise to a diagnostic potential in various clinical conditions such as transplantation and trauma.

Measurements of the change in NADH emission under UV (ultraviolet) excitation have been documented over the last 50 years in various organs including kidney, typically as a relative change of the signal intensity following an insult. The majority of these studies have used a small animal model such as rat [1-3], in which the capsule layer surrounding the kidney tissue is thin and its contribution to the total signal may be limited. However, the capsule of a human kidney can be up to several times thicker than that of a rat, and its contribution to the overall signal intensity through attenuation of the excitation light reaching the metabolically active cortex as well as generation of its own autofluorescence signal may need to be taken into account.

Despite the attention given to NADH fluorimetry, there has been no measurement of what proportion of the generated autofluorescence actually arises from the metabolically active kidney cortex tissue versus the capsule and how it is affected by the capsule's thickness. Such information would enable the quantitative correlation of the change in the observed autofluorescence intensity to changes in the NADH concentration in the cells associated with changes in the metabolism. At present, the experimental results, including a small number of cases on human kidney autofluorescence during transplantation [4], lack a quantitative analysis which would yield a more accurate description of the organ's metabolism and function. Signal quantification will enable the exploration of the full potential of this technology as an in vivo diagnostic tool to non-invasively obtain metabolic information in various clinical conditions such

as during surgical procedures associated with ischemic injury and organ response to reperfusion following traumatic injury or transplantation.

The objective of this work is to quantify the relative contribution in the measured autofluorescence from the cortex tissue compared to that from the capsule layer in the kidney under UV excitation using 355 nm and 266 nm as representative excitation wavelengths. Experimental results were obtained using a rat model, and a mathematical model was used to estimate the corresponding values in a human kidney.

Rat kidneys were excised and then kept in isotonic saline at 4˚C for 48 hours to allow an equilibrium in the NADH concentration to be reached after removal (due to change in metabolism) [5-6]. Measurements were successfully carried out on 7 kidneys total obtained from Wistar-Furth (2 kidneys, two rats), Sprague-Dawley (3 kidneys, two rats), and Lewis (2 kidneys, one rat) rats. Images of intact kidney, kidney with capsule removed, and one and two capsule layers without kidney, were acquired. Kidney with no capsule layer was prepared by cutting the capsule along one edge and pulling back to expose the cortex. Double capsule layer was formed by folding a single capsule layer back onto itself.

Two diode-pumped UV lasers were used for the photo-excitation of the kidney tissue operating at 355 nm and 266 nm delivering a dosage to the tissue of 0.10 mJ/mm^2 and 0.20 mJ/mm^2, respectively. Exposure time for each source was 5 seconds concurrent with image acquisition. Autofluorescence microscopic images were acquired using a microscopy system that has been described in detail elsewhere [7]. In brief, a liquid nitrogen-cooled CCD (charge-coupled device) camera (Roper Scientific, Princeton, New Jersey) was used to record the images which were formed using a 20X objective (Mitutoyo, Japan) followed by a 5X zoom lens after passing through a 420 nm long-pass filter. Each image was normalized to the fluorescence image

of a quartz slide to account for variation in beam intensity over illumination spot. Averages of 3-4 tubule regions near the center of the image were taken within a particular region as well as over these multiple regions.

Figure 1 shows typical autofluorescence images under 266 nm and 355 nm excitation of a rat kidney with its surface in three different configurations. Specifically, the images in Figs. 1a and 1c contain intact kidney on the top half of the image and exposed cortex on the bottom half under 266 nm and 355 nm excitations, respectively. Images shown in Figs. 1b and 1d contain an additional layer of capsule on the upper half (folded single capsule layer). Features of the tubules are visible in the bottom half of each image. Under 266 nm excitation, the morphology of the tubules is partially visible beneath one layer of capsule (a), but much less so under two capsule layers (b). The tubule morphology is visible in the corresponding images under 355 nm even through two capsule layers. The autofluorescence intensities for the different capsule configurations, normalized to the intensity from one capsule and averaged over the 7 kidneys, are shown in Table I.

The capsule thickness a was estimated from the image shown in Fig. 1a (and corresponding images from all other kidneys) and by measuring the distance in the axial direction needed to translate the kidney in order to move the image focus from the top of the capsule to the top of the cortex. The rat kidney capsule thickness was measured to be $a = 25 \pm 5\mu m$ for all 7 kidneys.

Due to difficulty in straightening out the entire capsule layer(s), each measured intensity is an average over a region of interest of about 25 μm × 25 μm to avoid folds. Factors contributing to the observed standard deviations in the experimental results may be attributed in

part to air gaps after capsule manipulation and in part to the presence of capsule heterogeneities such as small fatty or connective tissue residues that may have clung to the capsule.

A reasonable description of light propagation in turbid media can be obtained using simplified models [8]. Within such a formulation, the light intensity decreases exponentially ~Exp(-μx) with tissue depth x, where the *inverse* attenuation length (also the attenuation coefficient) μ is given by the combination of the absorption length μ_a^{-1}, scattering length μ_s^{-1}, and scattering anisotropy g (average scattering angle cosine) as $\mu=\mu_a+(1-g)\mu_s$. Since attenuation occurs for the incident light I_0 as well as for the fluorescence emission, the *effective* attenuation coefficient is the sum of the coefficients for incident and fluorescent light. Disregarding reflections at the tissue–air boundary, we can estimate the autofluorescence signal F from kidney containing capsule of thickness a over the cortex tissue, as

$$F = Q_C \int_0^a I_0 e^{-\mu_C x} dx + Q_T \int_0^\infty I_0(a) e^{-\mu_T x} dx = \frac{Q_C}{\mu_C} I_0 (1 - e^{-\mu_C a}) + \frac{Q_T}{\mu_T} I_0 e^{-\mu_C a} \quad (1)$$

where Q is a constant describing the emission efficiency per unit area of the tissue and indices C and T refer to capsule and tubules in cortex, respectively. The proportion of the signal from the cortex is given by

$$\frac{F_T}{F_C + F_T} = (1 + \frac{B}{\mu_C a} \times \frac{1 - e^{-\mu_C a}}{e^{-\mu_C a}})^{-1} \quad (2)$$

where the dimensionless constant $B = (Q_C \mu_T a)/Q_T$. B (which is proportional to the ratio of fluorescence conversion efficiency of capsule to that of cortex) and μ_C correspond to optical properties of the capsule and cortex and are independent of setup illumination and collection geometry.

By folding the isolated capsule and additionally making a measurement of the fluorescence signal with double capsule thickness $2a$, the capsule attenuation coefficient μ_C and the parameter B can be measured from Eqn. 2 for each incident wavelength by using a mean value of a=25 μm and the mean intensity values from the images (Table I). These constants were then used to plot the estimated dependence of the contribution of the cortex autofluorescence to the total signal (Eqn. 2) for arbitrary capsule thickness in Fig. 2, where the value for contribution of the tubules at 0 μm capsule thickness was defined as 100%. Values at 25 and 50 μm were obtained from the intensities from 1 and 2 capsule layers, respectively.

Based on direct measurements [9-10] and histological results [11], we assumed the average human kidney capsule thickness to be on the order of 100 μm. In addition, the human kidney capsule has a similar composition and structure to that of the rat kidney capsule comprised of dense connective tissue primarily of collagenous fibrils embedded with elastin fibers [12]. Therefore, we postulate that the results obtained from rat kidneys can be used to estimate the corresponding autofluorescence signal intensities in human kidneys. A previous study of the attenuation coefficient of collagenous tissue reported values from 50-500 cm^{-1} over an excitation range of 248-532 nm [13], consistent with our results. Based on the experimental results and the mathematical model, the percent contribution of the cortex to the total detected autofluorescence intensity as a function of capsule thickness under 266 nm and 355 nm excitations (averaged over the 7 kidneys) is shown in Fig 2. Standard deviation at 25 and 50 μm are based on mean values from the measurements. Standard deviations for values at thickness > 50 μm are based on mean values from the modeling results. The values at 100 μm represent the estimated values for a human kidney.

The results suggest that about half of the detected autofluorescence in a human kidney under 355 nm excitation would arise from the cortex, contributed to partially by NADH and thus carrying cellular metabolic information. This finding also enables for signal quantification to correlate measured changes in signal intensity (by rescaling the intensity accordingly) to changes in cellular NADH concentration arising from disruptions in tissue metabolism commonly encountered in the clinical settings previously described.

For signal quantification, it is also necessary to account for changes induced by variable illumination-collection geometry and changes in the absorption by the blood (arising from changes in the oxygenation level or blood volume). There have been several methods proposed to address this issue. One approach is to subtract the scattered excitation light signal from the fluorescence signal [2]. This method can be accurate only when the probe is in contact with the tissue but can give rise to additional signal artifacts, such as localized variations from the presence of microvasculature. Another approach is to monitor reflectance near hemoglobin isosbestic wavelengths in the near-IR region [1]. However, this method probes deeper into the kidney than does the UV excitation and may monitor blood volume changes occurring beneath the kidney cortex where hemodynamic properties are known to differ.

Using a non-contact optical probe, the amount and angular distribution of the reflected light contains an unknown mixture of diffuse and specular components which can change with illumination-collection geometry and hydration state, potentially creating signal artifacts independent of tissue metabolism when the organ is exposed during clinical procedures. The fact that 266 nm excitation would primarily be confined to the human capsule (~12% of signal would come from cortex) can be used to address these scattering changes. Since the emission under excitation near 266 nm arises mainly from biomolecules not involved in metabolism such as

tryptophan and collagen, this emission should only depend on probe-tissue geometry and specular reflection changes corresponding to variable hydration state [14]. Additionally, the capsule would effectively screen the 266 nm light which in sufficient doses can be harmful to cortex tissue [15]. Blood absorption would have to be estimated by another method, such as analyzing the strength of blood absorption features in the broad NADH emission spectrum.

In conclusion, the results presented in this work address the issue of signal quantification of NADH autofluorescence as a tool to noninvasively obtain metabolism and function-related information from kidneys. In vivo studies will help refine and validate the methods discussed in this work.

Acknowledgments

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References

1. S. Kobayashi, K. Kaede, K. Nishiki, and E. Ogata, "Microfluorometry of oxidation-reduction state the rat kidney in situ," *J. Appl. Physiol.* **31**(5), 693-696 (1971).
2. A. Mayevsky and B. Chance, "Intracellular oxidation-reduction state measured in situ by a multichannel fiber-optic surface fluorometer," *Science* **217**(4559), 537-540 (1982).

3. J. M. C. C. Coremans, M. Van Aken, D. C. W. H Naus, M. F. Van Velthuysen, H. A. Bruining and G. J. Puppels, "Pretransplantation assessment of renal viability with NADH fluorimetry," *Kidney Int.* **57**(2), 671-683 (2000).
4. A. Mayevsky, J. Sonn, M. Luger-Hamer, and R. Nakache, "Real-time assessment of organ vitality during the transplantation procedure," *Transplant Rev* **17**(2)**,** 96-116 (2003)
5. G. M. Palmer, C. L. Marshek, K. M. Vrotsos, and N. Ramanujam, "Optimal methods for fluorescence and diffuse reflectance measurements of tissue biopsy samples," *Laser Surg. Med.* **30**(3), 191-200 (2002).
6. K.T. Schomacker, J. K. Frisoli, C. C. Compton, T. J. Flotte, J. M. Richter, N. S. Nishioka, and T. F. Deutsch, "Ultraviolet laser-induced fluorescence of colonic tissue: basic biology and diagnostic potential," *Laser Surg. Med.* **12**(1), 63-78 (1992).
7. S.G. Demos, C.A. Lieber, B. Lin, and R. Ramsamooj, "Imaging of tissue microstructure using a multimodal microscope design," *IEEE J. Sel. Top. Quant.* **11**(4) 752-758 (2005).
8. S. Jacques, "Simple optical theory for light dosimetry during PDT," *Proc. SPIE* **1645**, 155-165 (1992).
9. J. G. Snedeker, P. Niederer, F. R. Schmidlin, M. Farshad, C. K. Demetropoulos, J. B. Lee, and K. H. Yang, "Strain-rate dependent material properties of the porcine and human kidney capsule," *J. Biomech.* **38**(5), 1011-1021 (2005).
10. H. Yamada. *Strength of biological materials,* G. Evans, ed. (Williams and Wilkins, Huntingdon, NY 1970).
11. L. Weiss, ed., Cell and tissue biology: a textbook of histology, 6th ed. (Urban and Schwarzenberg, Baltimore, 1988).

12. R. E. Bulger, “Rat renal capsule: presence of layers of unique squamous cells,” *Anat. Rec.* **177**(3), 393-403 (1973).

13. F.W. Cross, R.K. Al-Dhahir, and P.E. Dyer, “Ablative and acoustic response of pulsed UV laser-irradiated vascular tissue in a liquid environment,” *J. Appl. Phys.* **64**(4), 2194-2201 (1988).

14. J. T. Fitzgerald, A. P. Michalopoulou, C. D. Pivetti, R. N. Raman, C. Troppmann, and S. G. Demos, “Real-time assessment of *in vivo* renal ischemia using laser autofluorescence imaging,” *J. Biomed. Opt.* **10**(4), 044018 (2005).

15. Y. Matsumura and H. N. Ananthaswamy, “Short-term and long-term cellular and molecular events following UV irradiation of skin: implications for molecular medicine,” *Expert Rev. Mol. Med.* **4**, 1-22 (2002).

Figure 1

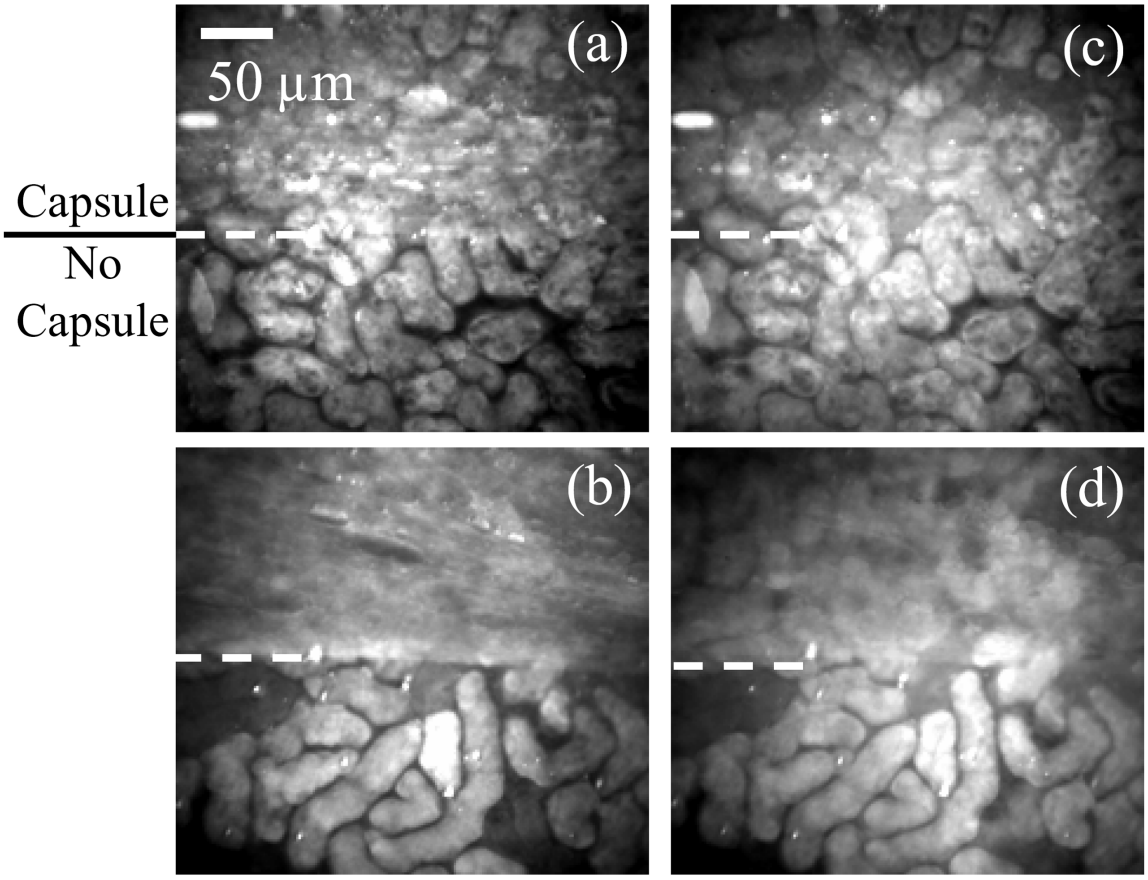

Figure 2

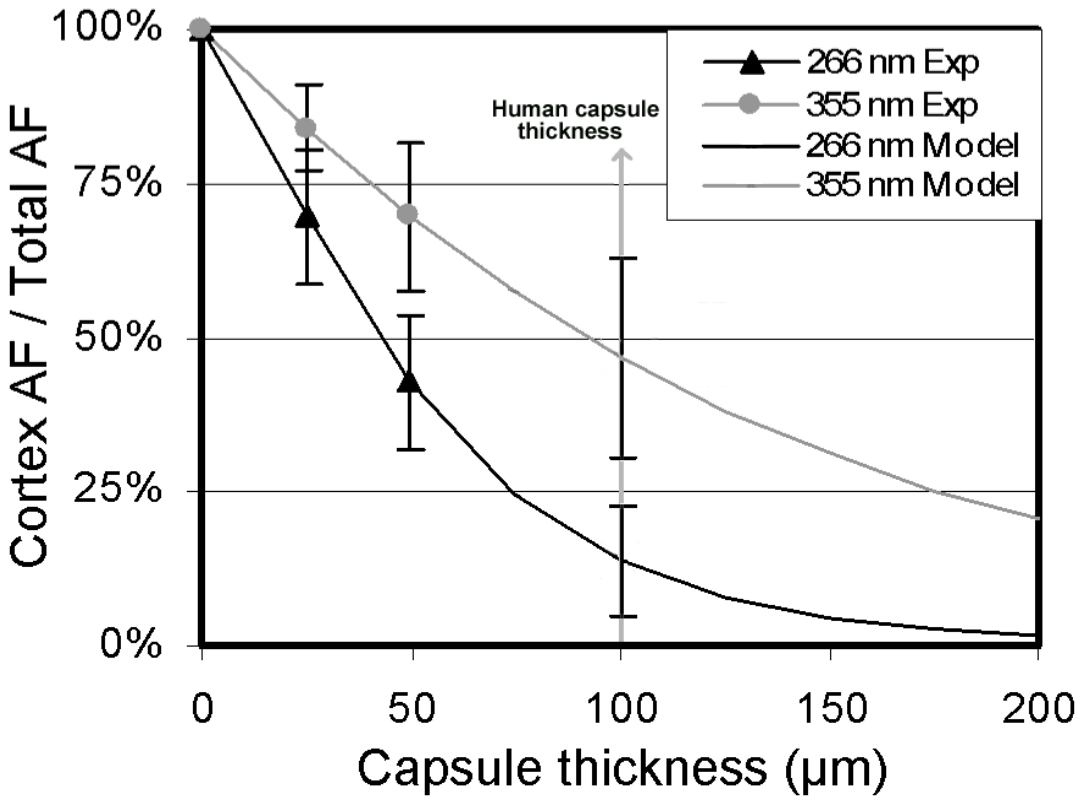
100%
75%
50%
25%
0%
Cortex AF / Total AF
0
50
100
150
200
Capsule thickness (μm)
Human capsule thickness
266 nm Exp
355 nm Exp
266 nm Model
355 nm Model

Table I. Autofluorescence intensities (mean ± SD for n=7 rat kidneys) normalized to that of 1 capsule layer. Values for μ_C (cm^{-1}) and B were calculated from these intensities according to the model described in the text.

	266 nm	**355 nm**
1 Capsule Layer Only	1.00	1.00
2 Capsule Layers Only	1.64 ± 0.20	1.86 ± 0.14
1 Capsule Layer on Kidney	3.63 ± 1.24	7.30 ± 2.91
2 Capsule Layers on Kidney	3.03 ± 0.90	6.81 ± 2.09
μ_C (cm^{-1})	326 ± 154	116 ± 54
B	0.300 ± 0.133	0.168 ± 0.082

Figure Captions

1. Fig. 1. Autofluorescence microscopy images of *ex vivo* rat kidney with capsule removed from lower half of each image. Images (a) and (b) are under 266 nm excitation while images (c) and (d) are under 355 nm excitation. Images (b) and (d) contain an additional layer of capsule on the upper half of the kidney.

2. Fig. 2. Plots of the percent contribution of the cortex to the total detected autofluorescence signal versus capsule thickness (mean values, n=7) under 266 nm and 355 nm excitations. Arrow indicates estimated values for human kidney. Standard deviation bars are indicated at 25, 50, and 100 μm capsule thicknesses. *AF*=autofluorescence intensity, *Exp*=experimental